微生物学通报
Microbiology China
tongbao@im.ac.cn

Jul. 20, 2013, 40(7): 1241–1248

简　报

A pSET152 derivative vector marked with thiostrepton resistance gene for introducing DNA into *Streptomyces*

DENG Ming-Rong　GUO Jun　FENG Guang-Da　ZHU Hong-Hui*

(*State Key Laboratory of Applied Microbiology, South China (The Ministry-Province Joint Development), Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Open Laboratory of Applied Microbiology, Guangdong Institute of Microbiology, Guangzhou, Guangdong* 510070, *China*)

Abstract: [Objective] In the PCR-targeting system of *Streptomyces*, apramycin is the most widely used selection marker. However, using apramycin as selection marker at disruption stage precludes, during subsequent genetic complementation, the use of some very useful and widely used vectors with the same marker, such as pSET152. This often caused unwanted inconvenience, especially in the situation that the physiological function of interested gene is sensitive to gene dosage, such as regulatory genes. The current study aims to provide an integrative plasmid with selection marker rather than apramycin. **[Methods]** Fusion-PCR and λ Red recombination were adapted to construct the new vector. **[Results]** The *bla* gene from pCR2.1 and the *tsr* gene from pHZ1358 were fused in the *tsr-bla* order. This fused fragment replaced the *aac(3)-IV* gene on pSET152 to generate pGIM6626. This new vector was validated by successfully restoring granaticin production of a granaticin-deficient *S. vietnamensis* mutant by re-introducing the deleted minimal polyketide synthase genes. **[Conclusion]** We constructed a new pSET152 derivative vector, pGIM6626, which contains ampicillin and thiostrepton resistance genes for selection in *E. coli* and *Streptomyces*, respectively. pGIM6626 and pSET152 have similar uses, but the former is more compatible with the PCR-targeting system because of no conflict of selection marker.

Keywords: Streptomyces, Integrative vector, pSET152, Thiostrepton resistance gene, PCR-targeting

Foundation item: National Natural Science Foundation of China (No. 31100042); Guangdong Natural Science Foundation (No. S2011010001625); Guangzhou Pearl River Rising Star Program for Science and Technology (No. 2013091); Guangdong Chinese Academy of Science Comprehensive Strategic Cooperation Project (No. 2012B091100276)
***Corresponding author:** Tel: 86-20-87137669; ✉: zhuhh@gdim.cn
Received: September 6, 2012; **Accepted:** November 14, 2012

一个硫链丝菌素抗性基因标记、用于 DNA 导入链霉菌的 pSET152 衍生载体

邓名荣 郭俊 冯广达 朱红惠*

(广东省微生物研究所 省部共建华南应用微生物国家重点实验室 广东省菌种保藏与应用重点实验室 广东省微生物应用新技术公共实验室 广东 广州 510070)

摘 要: 【目的】在链霉菌 PCR-targeting 系统中，安普霉素是使用最普遍的选择标记。然而在基因敲除阶段利用安普霉素选择标记后，在遗传补偿时就不能使用相同选择标记的许多重要载体，如 pSET152。这常给研究带来不便，特别是当研究对象基因如一些调控基因，其生理功能对剂量敏感时更是如此。基于此，拟以 pSET152 为基础构建一个不以安普霉素为抗性标记的通用整合型载体。【方法】利用融合 PCR 和 λ Red 重组等方法构建载体。【结果】来自 pHZ1358 上的硫链丝菌素抗性基因 *tsr* 和来自 pCR2.1 的氨苄抗性基因 *bla* 以“*tsr* 在前 *bla* 在后”的次序融合。融合后的抗性片段替换 pSET152 上的安普霉素抗性基因 *aac(3)-IV*，从而获得新载体 pGIM6626。利用该载体将删除的榴菌素最小聚酮合酶基因重新导入到 *Streptomyces vietnamensis* 突变株中，该突变株恢复了产榴菌素的能力，证实了该载体的有效性。【结论】构建了一个新的 pSET152 衍生载体 pGIM6626。该载体包含氨苄和硫链丝菌素抗性基因，分别在大肠杆菌和链霉菌中作为选择标记。pGIM6626 与 pSET152 用途相似，但前者由于与 PCR-targeting 系统不存在选择标记的冲突而与该系统更兼容。

关键词: 链霉菌，整合型载体, pSET152，硫链丝菌素抗性基因, PCR-targeting 系统

1 Introduction

The genetics of *Streptomyces* has received much attention in the last few decades because of the growing interests in understanding the mechanisms underlying their complex life cycles and the biosynthesis of their secondary metabolites. Gene disruption is one of the most important and efficient strategies to unveil the physiological roles of interested genes. In this strategy, a subsequent functional complementation experiment is routinely required to exclude any possible deleterious effect on other genes caused by the disruption itself. For gene disruption, many vectors and other genetic tools have been developed. Particularly, the adaptation to *Streptomyces* of λ Red mediated recombination (called PCR-targeting system) has much facilitated the generation of specific mutants[1]. This technology has since become a preferred disruption method. For gene complementation, there are two types of plasmids available: the auto-replicating plasmids and the integrative plasmids. However, when the physiological function of interested gene is sensitive to gene dosage, such as regulatory genes, the integrative plasmids are more feasible. Unfortunately, the integrative plasmids are relatively rare and most of which are apramycin resistant, such as the widely used pSET152[2]. In the

PCR-targeting system, apramycin is also the most widely used selection marker, though a serial of disruption cassettes containing different resistance markers have been constructed by the establisher. This is because, like the thiostrepton resistance gene *tsr*, the apramycin resistance gene *aac(3)-IV* provides very clean selection at single copy[3]. In the PCR-targeting system, although reusing the same selection marker both in disruption and complementation stages can be achieved by excision of the resistance gene before complemental experiment, removal of the resistance gene is often laborious, and sometimes impossible because the recombination efficiency of FLP recombinase varies markedly in different *Streptomyces* strains.

To provide an integrative plasmid with selection marker rather than apramycin, we set out to construct a pSET152 derivative, pGIM6626, which contains thiostrepton and ampicillin resistance genes. This novel plasmid was validated by restoring granaticin production in a granaticin-deficient mutant of *S. vietnamensis*.

2 Materials and methods

2.1 Plasmids, strains, primers and culture conditions

All plasmids, strains and primers used in this study are as described in Table 1. Luria-Bertani (LB) medium and SOB medium were used for growing *Escherichia coli* strains[4]. For induction of the expression of λ Red genes, L-arabinose (10 mmol/L final concentration) was added to SOB medium from a 1 mol/L filter-sterilized stock solution. *S. vietnamensis* strains were grown at 30 °C on Gauze's synthetic agar for spore preparation or in YEME liquid for mycelium growth[3]. YD agar medium was used to screen exconjugant during the intergeneric conjugation experiment[5]. Ampicillin (50 mg/L), apramycin (50 mg/L), chloramphenicol (25 mg/L), kanamycin (50 mg/L) or thiostrepton (15 mg/L) were added to growth media when required.

2.2 Construction of pGIM6626

General DNA manipulations were carried out according to standard procedures[4]. DNA from agrose gel was purified using a gel extraction kit (SK8142, Sangon, Shanghai, China). Plasmid pHZ1358 and pCR2.1 were digested with *Eco*R I/ *Hin*d III and *Eco*R I, respectively. Then the fragments containing the *tsr* gene (from pHZ1358) or the *bla* gene (from pCR2.1) were recovered and used as templates. Primer pairs Red-tsrF/TsrR and Tsr-ampF/Red-ampR (listed in Table 1) were respectively designed for amplification of the *tsr* and the *bla* genes, and reactions were performed with KOD DNA polymerase (Code KOD-101, Toyobo, Japan). PCR conditions for amplification of the *bla* gene were as follows: predenaturation at 95 ℃ for 3 min, then 7 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 ℃ for 40 s, followed by 23 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 40 s, with a final extension at 72 °C for 10 min. For amplification of the *tsr* gene, the annealing temperature of the first 7 cycles and the subsequent 23 cycles were changed to 57 °C and 62 °C, respectively. The amplified marker genes were purified and subjected to a PCR-fusion procedure. The amplified *bla* cassette overlaps 29 bp at the 5′ end with the 3′ end of the *tsr* gene, which allows these two PCR fragments to be ligated into one in the *tsr-bla* order by a PCR reaction. The reaction mixture without primers underwent an initial denaturation period at 96 °C for 3 min, followed by 6 cycles of 96 °C for 35 s, 58 °C for 50 s and 72 °C for 1 min, then was immediately cooled down to room temperature for 1 min to allow the primers Red-tsrF and Red-ampR to be added. The reaction mixture with primers continued to go through 24 cycles of 96 °C for 35 s, 62 °C for 50 s and 72 °C for 1 min, which followed by a final extension at 72 °C for 10 min.

The fused fragment (the *tsr-bla* cassette) was gel-purified, precipitated with ethanol, then dissolved in a minimum of sterile distilled water and used to replace the *aac(3)-IV* gene on pSET152. The replacement was mediated by λ Red recombinases. The basic procedure of recombination was as described by Gust et al[4], but with some modifications. Briefly, the pSET152 plasmid was transformed by the heat-shock method into chemically competent BW25113/pIJ790 cells. The resulting BW25113/pIJ790/pSET152 was electrically transformed with the purified *tsr-bla* cassette. Because

Table 1 Plasmids, strains and primers used in this study
表 1 本研究所用质粒、菌株和引物

Strain/Plasmid/Primer	Description	Reference/Source/Remark
Plasmids		
pSET152	*Aac(3)-IV*, *lacZα*, *rep*^{pUC}, *attp*$^{\varphi C31}$, *oriT*	[2]
pHZ1358	pIJ101 derivative; *tsr*, *Ltz*$^{-}$, *sti*$^{+}$, *oriT*	[6]
pCR2.1	*bla*, *neo*; TA cloning vector	Invitrogen
pUZ8002	*tra*, *neo*, RP4; helping plasmid for conjugation	[7]
pIJ790	λ-Red (*gam*, *bet*, *exo*), *cat*, *araC*, *rep101*^{ts}	[1]
pGIM6626	*tsr*, *bla*, *lacZα*, *rep*^{pUC}, *attp*$^{\varphi C31}$, *oriT*	This study
pGIM-pks	pGIM6626 inserted with the *gra-orf*1,2,3 fragment	This study
Strains		
S. vietnamensis		
GIMV4.0001	Wild type	[8]
DMR1	Δ*gra-orf1*,*2*,*3*::(*oriT-aac(3)IV*)	[9]
DMR1C	Δ*gra-orf1*,*2*,*3*::(*oriT-aac(3)-IV*), Δ*attp*:: *gra-orf1*,*2*,*3*	This study
E. coli		
JM109	*endA*1, *recA*1, *gyr*A96, *thi*-1, *hsdR*17(r_K–, m_K+), *relA*1, *supE*44, Δ(*lac-proAB*), [F', *traD*36, *proAB*, *lac*I^{q}ZΔM15]; general cloning strain	[10]
BW25113	Δ*araBAD*, Δ*rhaBAD*; for λ Red recombination	[11]
Primers		
Red-tsrF	TATGATCGACTGATGTCATCAGCGGTGGAGTGCAATGTCATGACTGAGTTGGACACCAT	39 nt (underlined) upstream of *aac(3)-IV* on pSET152
Red-ampR	CTTGCCCCTCCAACGTCATCTCGTTCTCCGCTCATGAGCTTACCAATGCTTAATCAGTG	39 nt (underlined) downstream of *aac(3)-IV* on pSET152
TsrR	TTATCGGTTGGCCGCGAGAT	Paired with Red-tsrF to amplify *tsr*
Tsr-ampF	TCGACAGGAATCTCGCGGCCAACCGATAAATTGAAAAAGGAAGAGTATG	29 nt (underlined) identical to the 3′ end of *tsr*, paired with Red-ampR to amplify *bla*
COMpksF	CTAGTCTAGACTCGTCTCCCTTGGGTTCCT	*Xba* I (underlined), for complementational plasmid construction
COMpksR	CCGGAATTCGGGCGTACTCCTTGGAC	*Eco*R I (underlined), for complementational plasmid construction
Gra-orf2F	GGGCCAGATCGACGACTTC	The PCR product spanning *gra-orf*2 and *gra-orf*3, for detecting the existence of the minimal PKS genes for granaticin
Gra-orf3R	GACGAGGATGGTGCGCAGTA	

there were 39 bp of homologies to the flanking regions of the *aac(3)-IV* gene on pSET152 at both ends of the *tsr-bla* cassette, the *aac(3)-IV* gene would be replaced by the *tsr-bla* cassette after induction of expression of λ Red genes. The ampicillin-resistant transformant was grown in liquid LB,

and subjected to plasmid extraction. The resulting plasmid preparation was a mixture of wild pSET152 and mutagenized pSET152 (i.e., pGIM6626). These mixed plasmids were transformed further into *E. coli* JM109. Plasmids extracted from the ampicillin-resistant transformants in this round were considered as pure pGIM6626, and were confirmed by restriction analysis.

2.3 Restoration of granaticin production of *S. vietnamensis* DMR1

S. vietnamensis DMR1 is a granaticin-deficient mutant with deletion of the minimal polyketide synthase (PKS) genes for granaticin[9]. In order to confirm its effectiveness, pGIM6626 was used to re-introduce the three minimal PKS genes, *gra-orf1*, *gra-orf2* and *gra-orf3*, into this mutant. Genomic DNA of *S. vietnamensis* was isolated as described elsewhere[3]. Primers COMpksF and COMpksR (listed in Table 1) were designed to amplify the three genes. Long PCR was performed using LA *Taq* DNA polymerase (DRR02AG, TaKaRa, China) as described previously[12]. The PCR product contained 241 bp upstream from the start codon of *gra-orf1*, the three minimal PKS genes and 131 bp downstream from the end of *gra-orf3*. The amplified fragment was 3 169 bp long and flanked by *Xba* I and *Eco*R I restriction sites at the upstream and downstream ends, respectively. This fragment was digested with *Xba* I and *Eco*R I, then recovered and ligated to *Xba* I/*Eco*R I-digested pGIM6626. The resulting plasmid pGIM-pks was confirmed by restriction analysis and sequencing, then introduced into *E. coli* ET12567/pUZ8002. The intergeneric conjugation between *E. coli* ET12567/pUZ8002/pGIM-pks and *S. vietnamensis* DMR1 was done on YD agar medium as described previously[9]. Putative exconjugants were screened by thiostrepton. The putative complementants were verified by both phenotype and genotype analysis. The purified thiostrepton-resistant exconjugants were streaked onto Gauze's synthetic plate to test the ability to produce granaticin. The characteristic violet-blue color of granaticin was observed by naked eye. The strains producing granaticin were subjected to PCR validation. The specific primer pair Gra-orf2F/Gra-orf2R (listed in Table 1) was designed to detect the existence of the minimal PKS genes for granaticin.

3 Results and discussion

3.1 Construction of pGIM6626

Many *Streptomyces* species hold an *attB* locus in their chromosomes[3], therefore integrating vectors based on the φC31 *int*/*attP* are employed widely in the genetic analysis of *Streptomyces* species. The most widely used integrating vector, pSET152, uses apramycin as selection marker, which is also the most popular selection marker in the PCR-targeting system. To improve the compatibility between the integrating vectors and the PCR-targeting system, the thiostrepton resistance gene *tsr*, which is not used in the PCR-targeting system, was adopted to construct a novel pSET152-based vector. The construction scheme is presented in Fig. 1. The *tsr* gene was amplified from pHZ1358 by PCR (Fig. 2A). The resulting fragment is 849-bp long and the first 39 bp at the 5′ end are identical to the upstream sequence of the *aac(3)-IV* gene on pSET152. Because *E. coli* strains are not sensitive to thiostrepton, another resistance gene must be included to allow DNA manipulation in *E. coli*. A fragment from pCR2.1 containing the ampicillin resistance gene *bla* and 17 bp upstream from the start codon of *bla* was amplified (Fig. 2A). The amplified 947-bp long fragment overlapped 29 bp at the 5′ end with the 3′ end of the *tsr* gene, and had a 39-bp homologous region at the 3′ end to the downstream sequence of the *aac(3)-IV* gene on pSET152. The 29-bp overlap allowed the amplified *tsr* and *bla* fragments to be ligated to form a *tsr-bla* cassette by a fusion PCR (Fig. 2A). This 1 767-bp cassette contained a 39-bp homologous region at each end to the flanking sequences of the *aac(3)-IV* gene on pSET152, and underwent a λ Red recombination process to replace the *aac(3)-IV* gene on pSET152. This new generated plasmid was predicted to possess ampicillin and thiostrepton resistance genes for selection in *E. coli* and *Streptomyces* spp., respectively. It was confirmed by restriction analysis (Fig. 2B), and thus was designated as pGIM6626.

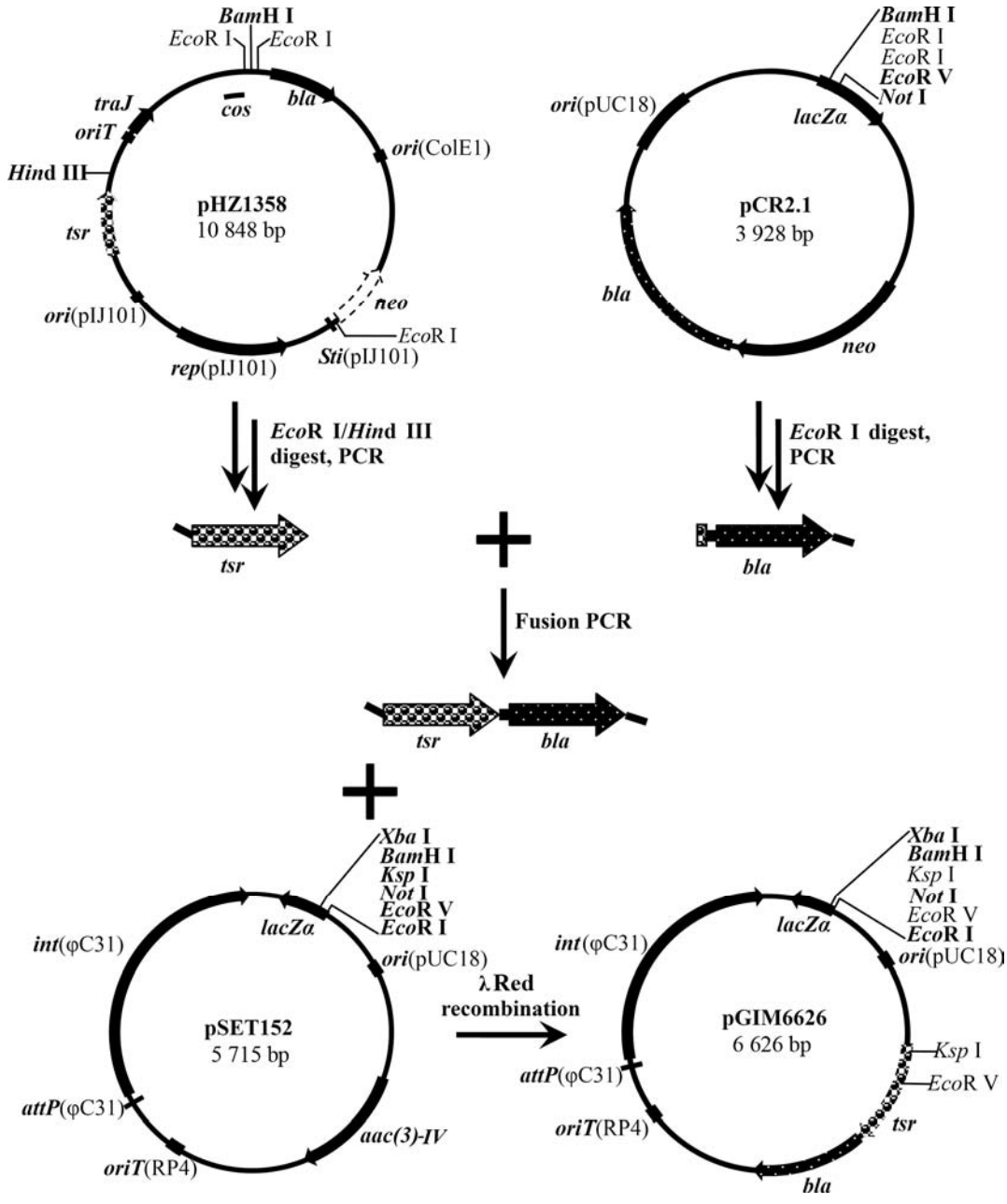

Fig. 1 Scheme for the construction of pGIM6626

图 1 pGIM6626 构建示意图

Note: The *tsr* and the *bla* genes were amplified from pHZ1358 and pCR2.1, respectively. These two amplified fragments were fused by PCR through a 29-bp-overlap between them. The resulting *tsr-bla* cassette had a 39-bp homologous region at each end to the flanking sequences of the *aac(3)-IV* gene on pSET152, then it was used to replace the *aac(3)-IV* gene on pSET152 by λ Red recombination to generate pGIM6626.

3.2 Restoration of granaticin production of *S. vietnamensis* DMR1

S. vietnamensis DMR1 was constructed previously by replacement of the minimal PKS genes for granaticin with the *aac(3)-IV* gene using the PCR-targeting system[9]. To restore granaticin production, the three minimal PKS genes, *gra-orf1*, *gra-orf2* and *gra-orf3*, were amplified from the wild-type genome of *S. vietnamensis* and inserted into pGIM6626 between *Xba* I and *Eco*R I sites.

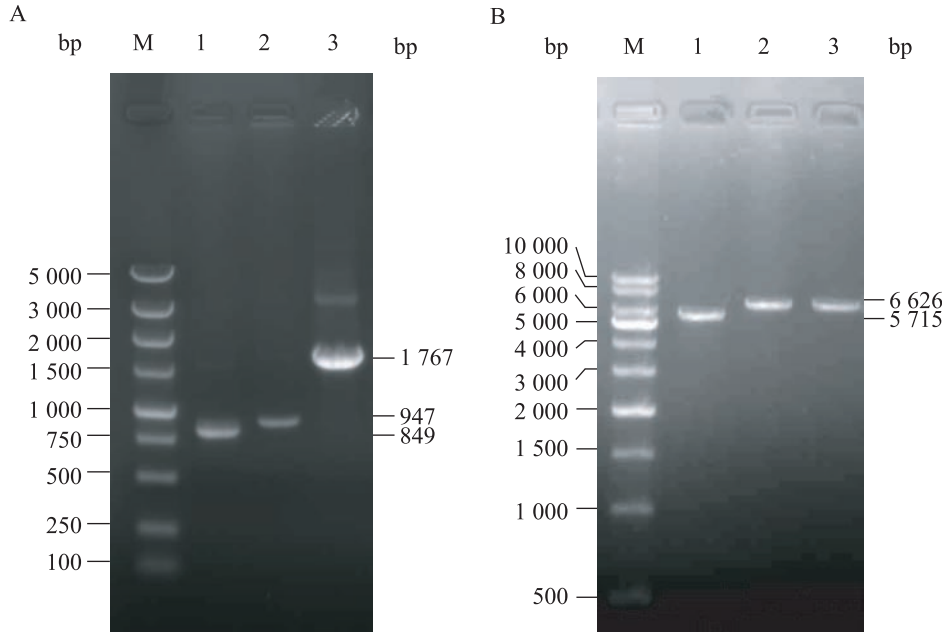

Fig. 2 The construction of pGIM6626

图 2 pGIM6626 构建电泳图

Note: A: Amplification of the *tsr* and the *bla* gene, and their fusion. M: DS5000 DNA marker; 1: *tsr* gene; 2: *bla* gene; 3: The fused *tsr-bla* cassette. B: Comfirmation of the successful construction of pGIM6626 by restriction analysis. M: 1 kb ladder DNA marker; 1: pSET152 digested with *Bam*H I; 2, 3: pGIM6626 digested with *Bam*H I.

The successful complementation of the minimal PKS genes was confirmed by PCR and observation of granaticin production. As shown in Fig. 3A, the minimal PKS genes were detected in the complemented strain DMR1C. The secretion of violet-blue pigment of DMR1C on Gauze's synthetic plate showed clearly that this strain restored granaticin production (Fig. 3B).

4 Conclusion

We have constructed a new pSET152 derivative vector, pGIM6626, which contains ampicillin and thiostrepton resistance genes for selection in *E. coli* and *Streptomyces*, respectively. pGIM6626 and pSET152 have similar uses in the genetic analysis of *Streptomyces* species. But pGIM6626 is more compatible with the PCR-targeting system because of no conflict of selection marker.

Acknowledgments

The authors are grateful to Prof. Zi-Xin Deng and Prof. De-Lin You at Shanghai Jiao Tong University for providing the strain ET12567/pUZ8002 and the plasmids pSET152 and pHZ1358.

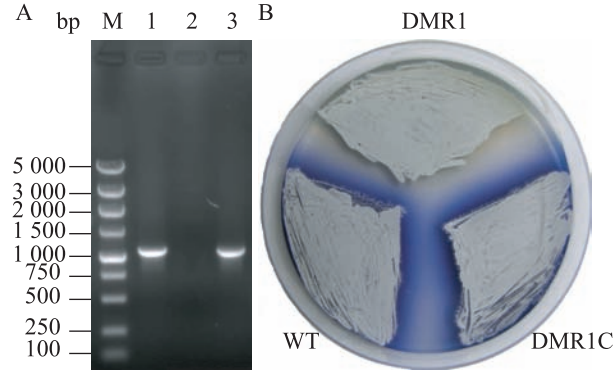

Fig. 3 Restoration of granaticin production of *S. vietnamensis* DMR1

图 3 *S. vietnamensis* DMR1 恢复生产榴菌素

Note: A: Detection of the existence of the minimal polyketide synthase (PKS) genes in the genome of the complemented strain DMR1C. M: DS5000 DNA marker; 1: *S. vietnamensis* wild type; 2: *S. vietnamensis* DMR1 (granaticin-deficient mutant); 3: *S. vietnamensis* DMR1C (the complemented strain). B: *S. vietnamensis* wild type (WT), DMR1 and DMR1C grown on Gauze's synthetic agar. The cultures were 3 d old. The granaticin-deficient mutant DMR1 didn't produce any blue pigment (granaticin), while the complemented strain DMR1C restored granaticin production near to the level of wild type.

REFERENCES

[1] Gust B, Challis GL, Fowler K, et al. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin[J]. Proceedings of the Nationsl Academy of Sciences of the United States of America, 2003, 100(4): 1541−1546.

[2] Bierman M, Logan R, O'Brien K, et al. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp.[J]. Gene, 1992, 116(1): 43−49.

[3] Kieser T, Bibb MJ, Buttner MJ, et al. Practical *Streptomyces* Genetics[M]. 2nd ed. Norwich: John Innes Foundation, 2000: 1−613.

[4] Sambrook J, Russell DW. Molecular cloning: a laboratory manual[M]. 3rd ed. New York: Cold Spring Harbor Laboratory, 2001: 1−999.

[5] Foor F, Roberts GP, Morin N, et al. Isolation and characterization of the *Streptomyces cattleya* temperate phage TG1[J]. Gene, 1985, 39(1): 11−16.

[6] Sun YH, Zhou XF, Liu J, et al. '*Streptomyces nanchangensis*', a producer of the insecticidal polyether antibiotic nanchangmycin and the antiparasitic macrolide meilingmycin, contains multiple polyketide gene clusters[J]. Microbiology, 2002, 148(Pt 2): 361−371.

[7] Paget MSB, Chamberlin L, Atrih A, et al. Evidence that the extracytoplasmic function sigma factor sigma E is required for normal cell wall structure in *Streptomyces coelicolor* A3(2)[J]. Journal of Bacteriology, 1999, 181(1): 204−211.

[8] Zhu HH, Guo J, Yao Q, et al. *Streptomyces vietnamensis* sp. nov., a streptomycete with violet-blue diffusible pigment isolated from soil in Vietnam[J]. International Journal of Systematic and Evolutionary Microbiology, 2007, 57(8): 1770−1774.

[9] Deng MR, Guo J, Zhu HH. *Streptomyces vietnamensis* GIMV4.0001: a granaticin-producing strain that can be readily genetically manipulated[J]. The Journal of Antibiotics, 2011, 64(4): 345−347.

[10] Yanisch-Perron C, Vieira J, Messing J. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUC19 vectors[J]. Gene, 1985, 33(1): 103−119.

[11] Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products[J]. Proceedings of the National Academy of Sciences of the United States of America, 2000, 97(12): 6640−6645.

[12] Deng MR, Guo J, Li X, et al. Granaticins and their biosynthetic gene cluster from *Streptomyces vietnamensis*: evidence of horizontal gene transfer[J]. Antonie van Leeuwenhoek, 2011, 100(4): 607−617.